THE PRESENCE - ABSENCE PROCEDURE

Ontario
Ministry of the Environment

The Honourable
George A. Kerr, Q.C.,
Minister

K.H. Sharpe,
Deputy Minister

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The Presence-Absence Procedure for Detection of Pollution Indicator Bacteria in Drinking Water Supplies

The methodology for detection and enumeration of pollution indicator bacteria by membrane filtration has been described elsewhere. In this section, a methodology is described for the detection of these organisms by the presence-absence (P-A) test. This test has proven more sensitive for detecting pollution indicator bacteria in drinking water samples than the membrane filter (MF) procedure. Essentially the P-A test is a modification of the most probable number (MPN) procedure, which uses a dilution series of broth tubes to detect and enumerate microorganisms. The P-A test employs only one analysis bottle per sample to determine a wide variety of pollution indicator bacteria.

1.) Sample Handling and Preservation

Bacteriological samples must be collected in Ministry of the Environment (MOE) sterilized glass bottles. The bacteriological examination of a water sample should be initiated immediately after collection. However, as this is seldom practical, more realistic arrangements must be established. The sample bottle should be chilled (not frozen) and transported to the laboratory in 24 hours. In any event, bacteriological analyses are not done on samples aged four days or more. For specific programs, samples must arrive at the laboratory within 24 hours. Samples containing chlorine as a disinfecting agent should be collected in sterile bottles to which sodium thiosulphate ($Na_2S_2O_3$) has been added to neutralize any chlorine present.

2.) Selection of Method

Municipalities require analysis of their raw or source water, as well as the water at various stages of the treatment process in the plant and also after the water has left the plant and entered the distribution system. The laboratory analyzes samples of these waters by either the presence-absence (P-A) or membrane filter (MF) technique, or, in some instances, a combination of both techniques:

a) Raw water samples will be analyzed by the MF procedure for total coliforms (TC), fecal coliforms (FC), and fecal streptococci (FS), when TC, FC and FS counts are 10 or more per 100 ml based on previous analyses. If counts are less than 10 for any of the indicator organisms, a P-A test should be performed along with the MF test for total coliforms.

b) Plant samples will be analyzed for total coliforms and background counts by the MF test unless the enumeration of other indicator organisms is deemed necessary or requested. A P-A test will be included as part of the analysis for these samples.

ALOT

c) Distribution samples will be initially examined by a combination of MF and P-A tests. After establishing that these samples usually give negative results with the MF test, all samples will receive P-A tests and periodic random samples will be picked out and given both MF and P-A tests. This will be done usually on a one in four to one in eight basis, for the purpose of checking on background counts as well as possible total coliform counts. Any sample locations previously having pollution indicator organisms detected by the P-A test will receive an MF/P-A test combination on their next submission. Samples consecutively positive by both MF and P-A tests may be analyzed by only MF tests.

Presence-Absence Test Analysis

Summary

Organisms Determined	The test is designed to qualitatively detect a variety of pollution indicator organisms in water, including total coliforms, fecal coliforms, fecal streptococci, Pseudomonas aeruginosa, Staphylococcus aureus, Aeromonas sp. and Clostridium perfringens.
Interprepation of Results	The presence of any of the above groups represents inadequately treated and possibly hazardous water. For more detailed information, consult the MOE's Drinking Water Objectives.
Principle of Methods	Each of the above pollution indicator organisms may be present alone or in combination with each other. A selection of confirmatory and taxonomic tests permits differentiation of the bacteria present.
Time Required for Analysis	The initial analysis time is less than one minute per sample. Time required for presumptive results ranges from one to five days. Time required for confirmatory results is from one to four days depending on the pollution indicator group present.
Range of Application	Maximum sample volume for which an analysis is usually done is 100 ml; the minimum sample volume is usually 50 ml.
Standard Deviation	Performance characteristics are not applicable.
Accuracy	Performance characteristics are not yet available.
Limit of Detection	Performance characteristics are not yet available.
Shortcomings and Interferences	Because the P-A test is qualitative in nature, samples giving a positive test for pollution will require a MF or MPN analysis to determine numbers of organisms. Analysis of sample volumes greater than 100 ml will also require either MF or MPN methods.

Minimum Volume of Sample	160 ml of sample is preferred particularly if both MF and P-A analyses may be requested for the sample.
Preservation and Sample Container	MOE 175 ml sterile glass bottles should be used for collection of samples for drinking water analyses. Those for collection of chlorinated water samples must contain sodium thiosulphate to neutralize any chlorine present. Samples should be iced (not frozen) if possible during transportation to the laboratory and arrive within 24 hours of the sampling time.
Safety Considerations	Bacteriological samples, particularly those from sources of water suspected of pollution, may contain pathogenic organisms, and should be handled in a manner to prevent contamination of the sampler and the analyst.

Pollution Indicator Bacteria
Presence-Absence Analysis

1.) Introduction

The determination of total coliforms, fecal coliforms, fecal streptococci, Pseudomonas aeruginosa, Staphylococcus aureus, Aeromonas sp. and Clostridium perfringens bacteria by the presence-absence (P-A) test is performed by initially adding the water sample to a bottle containing the presumptive medium. Then, by a selection of confirmatory and taxonomic tests performed on a presumptive positive P-A bottle, any or all of the above groups of organisms may be isolated and identified. In some instances, total coliform and fecal coliform cultures may be isolated and identified at the generic level.

2.) Interference and Shortcomings

The identification of the different types of organisms present in water samples, particularly those representative of pollution indicator organisms, depends to a certain extent on their initial numbers in the original sample and their ability to grow and multiply in the mixed population existing in the presumptive P-A bottle. Following transfer to confirmatory media, which usually contain substances that preferentially select out certain organisms for growth and inhibit others, the isolation of particular organisms often depends on the skill of the technician to streak out mixed cultures on agar plates in order to obtain isolated, individual colonies. Sometimes, the isolation of particular types of organisms is made difficult because of the overgrowth on bacterial agar plates by other organisms present in larger numbers.

Other factors influencing the isolation of representative types of bacteria from the original sample will depend on storage conditions during transit to the laboratory and whether the sample contains any nutritive or inhibitory substances.

3.) Apparatus

a) Sterile Equipment

(i) pipettes - 1 ml

(ii) graduate cylinders - 100 ml for 100 ml volumes
- 50 ml for 50 ml volume

b) Non-sterile Equipment

(i) incubation racks for 6 oz and 8 oz bottles

(ii) one bunsen burner

(iii) inoculating loop(s) and needle(s)

(iv) wax crayon and marking pen for numbering P-A bottles, test tubes and petri dishes

(v) test tube rack for holding 40 test tubes

(vi) plastic "cakettes" for incubation of petri dishes

c) Accessory Equipment

(i) Waterbath and/or Incubators for maintaining temperatures at $\pm 0.5^{\circ}C$ in the ambient to $60^{\circ}C$ range

(ii) Stereoscopic microscoscope with 10X magnification for examining bacterial colonies

(iii) Cool white fluorescent light for illuminating bacterial colonies

(iv) Slanted (15°) wooden stage for resting petri dish during the examination of bacterial colonies

(v) Daylight fluorescent titration illuminator for examining bacterial growth in P-A bottles, test tubes and agar plates. A magnifying device with a built-in illuminator may also be used for examining and isolating bacterial colonies

(vi) Longwave ultraviolet (hand-held) light with wavelength of 366 nm

(vii) Laminar Flow Unit

4.) Reagents and Media used in the presence-Absence (P-A) test

a) Buffered Water - consists of 1000 ml of distilled water to which 1.25 ml of 0.25 M KH_2PO_4 (previously adjusted to pH 7.2 with 1N NaOH) is added. The buffered water is made up in dilution blanks and sterilized in an autoclave to result in a final volume of 99 ± 1 ml

b) Disinfectant for swabbing benches and disinfecting pipettes

(i) Wescodyne (1:30 with water)

(ii) Dettol (1:5 with water)

c) Reagents for Gram stain, Indole test and Oxidase test - see section 7 for preparation instructions

d) Media employed in the presence-absence (P-A) test

(i) 6 oz. P-A bottles - MacConkey broth

(ii) 8 oz. P-A bottles - MacConkey broth

(iii) EC broth

(iv) Drake's broth

(v) Ethyl Violet Azide (EVA) broth

(vi) Skim Milk broth

(vii) Tryptophane broth (Indol test)

(viii) Acetamide agar slants

(ix) MacConkey agar plates

(x) Enterococcus agar plates

(xi) Nutrient Gelatin agar plates

(xii) Skim Milk agar plates

(xiii) Mannitol Salt agar plates

(xiv) Arginine Dihydrolase medium

(xv) Phenol Red Dextrose broth

(xvi) OF Glucose medium

(xvii) Enterotubes (Hoffmann-La Roche - Roche Diagnostic Ltd.)

(xiii) Oxi/ferm tubes (Hoffmann-La Roche - Roche Diagnostic Ltd.)

(xix) Trypticase Soy broth

(xx) Trypticase Soy agar

With the exception of items (xvii) and (xviii), the instructions for preparation of the above media will be found in section 7

5.) A. Procedure - Presumptive Part of P-A Test

a) Samples must be kept refrigerated before analysis. Only samples which can be analyzed within one hour should be kept on the work bench. All others should be kept under refrigeration until they are required for analysis.

b) Preparation for the presence-absence (P-A) test

(i) Sterile technique must be employed throughout the analytical procedure

(ii) The work bench area is swabbed thoroughly with the Dettol solution and wiped dry

(iii) The samples are arranged on the work bench in numerical order according to the laboratory number

(iv) One 100 ml sterile graduate cylinder and one 8 oz P-A bottle are set up on the work bench beside each of the samples. Each P-A bottle is marked with the laboratory number corresponding to that on the sample bottle

(v) Before the P-A bottle containing the concentrated MacConkey broth is used for analysis, the medium in the inverted gas tube must be drained out.

This procedure is accomplished as follows:

(vi) Make sure the cap of the P-A bottle is securely tightened

(vii) Invert the P-A bottle until the gas tube falls into the neck of the P-A bottle

(viii) Slowly bring the P-A bottle to a horizontal position while keeping the gas tube in the neck of the P-A bottle by frictional contact

(ix) Very slowly bring the P-A bottle to an upright position allowing the medium in the gas tube to drain out before the gas tube is allowed to fall to the bottom of the P-A bottle

(x) As the gas tube falls back into the MacConkey medium, entrapped air keeps the concentrated medium from returning inside the tube until after the water sample has been added

(xi) The above procedure facilitates the complete mixing of the sample and the MacConkey broth, so that the latter becomes a single strength broth medium for cultivation of pollution indicator bacteria

c) Presumptive presence-absence (P-A) analysis

(i) The sample bottle is shaken vigorously 25 times, placed on the bench, and the cap loosened. The aluminum foil covering from a 100 ml graduate cylinder is removed and the mouth of the graduate cylinder is flamed

(ii) The sample bottle is picked up again and the cap of the bottle is held firmly by the fifth finger of one hand, while the bottle is unscrewed with the other hand held at the bottom of the bottle. The mouth of the bottle is flamed and 100 ml of sample is poured out into the 100 ml graduate cylinder. The mouth of the bottle is flamed again and the cap screwed back into place. During the above sequence, the cap of the sample bottle is held snugly by the fifth finger against the palm of the hand

(iii) The P-A bottle is picked up and the bottle cap is removed and held in the hand as described in the previous operation. The 100 ml sample in the graduated cylinder is poured into the P-A bottle; the mouth of the bottle is flamed; and the cap is screwed back into place. The P-A bottle is inverted four to five times to mix the sample and the growth (i.e. MacConkey broth) medium. The P-A bottle is left standing in an inverted position for 15-20 seconds to allow any gas bubbles introduced into the medium during the mixing procedure to come to the surface and dissipate. The bottle is turned right side up, allowing the gas tube to become filled with the inoculated medium and checked to see that no air bubbles have become trapped in the inverted tube

(iv) Each P-A bottle is transferred to a rack and placed in the $35^{o}C$ incubator for four to five days

d) Incubation period observations (P-A bottles)

(i) Each day for four to five days (samples analyzed on Wednesday have their last incubation day on the following Monday, i.e. 5 days), the P-A bottles are removed from the incubator and examined for any colour change or gas production. Depending on the degree and type of change, confirmatory tests are scheduled for presumptive positive P-A bottles

(ii) If pollution indicator organisms are present in the sample added to the P-A bottle, fermentation of the lactose sugar in the MacConkey medium will occur. This results in acidic conditions being produced and these conditions are monitored by a bromo cresol purple pH indicator in the MacConkey broth. As the medium becomes acidic, the colour changes from purple to yellow and the intensity of the change is subjectively recorded by the letters:

(a), a, (A), and A respectively, which represent weak to strong acid conditions

(iii) No change or no growth is recorded as O, and no colour change, but bacterial growth, is recorded as +

(iv) If the lactose fermentation is also accompanied by gas production, the amount of gas produced is recorded as: (g), g or G depending on the volume of gas trapped in the inverted tube. If sufficient gas is produced to fill the top curved portion of the inverted tube (approximate volume = 10%), the result is marked as G; if less than 10%, the result is marked as g; if only a trace of gas is present, i.e. small bubbles appear when the tube is tapped, the result is marked as (g)

(v) Any P-A bottle which has been scored as a, (A), A or (g), G or any combination of these symbols is removed from the rack for the performance of confirmatory tests

(vi) Any P-A bottle scored as O, + or (a) is left in the rack and the rack is returned to the 35°C incubator. These bottles are checked again every 24 hours until the end of their scheduled incubation period to determine whether further changes have taken place which would require confirmatory tests. If none of these tests is required on the bottles, they are discarded after four (or five) days. (Note: discarding of any bacterial analyses where growth of organisms has occurred, means that the discarded material is autoclaved before ultimately disposing of it)

5.) B. Procedure - Confirmatory analyses of the P-A test

a) The selection of confirmatory test analyses is dependent on the reaction occurring in the individual P-A bottle

(i) P-A bottles with (g), g or G reactions and no acid production receive the following tests:

Drake's tube (41.5°C) (96 h) — MacConkey agar plate (24 h)
Skim Milk tube (48 h) — Enterococcus agar plate (48 h)
Gram Stain

(ii) P-A bottles with a, (A) or A reactions and no gas production receive the following tests:

Drake's tube (41.5°C) (96 h) — MacConkey agar plate (24 h)
Ethyl Violet Azide (EVA) tube (48 h)
Gram stain — Enterococcus agar plate (48 h)
Mannitol Salt agar plate (48 h)

(iii) P-A bottles with any combination of both acid and gas reactions receive the following tests:

EC broth (48 h) — MacConkey agar plate (24 h)
EC broth (44.5°C) (48 h) — Enterococcus agar plate (48 h)
Drake's tube (41.5°C (96 h) — Mannitol Salt Agar plate (48 h)
Ethyl Violet Azide (EVA) tube (48 h) — Gram stain
Skim Milk tube (48 h)

(iv) All of the above confirmatory tests are incubated at 35°C unless otherwise indicated.

b) Method of inoculation, incubation period, type of result and additional testing required for the above confirmatory tests

(i) P-A bottles selected for confirmatory tests are divided into groups according to the types of tests required. All confirmatory media should be at room temperature before inoculation takes place and agar plates should be checked to insure the agar surface is dry before streaking with inoculum. Plates with moisture droplets can be placed in a 35°C incubator for 15-20 minutes or a laminar flow unit with the lids ajar to allow the air of the incubator or laminar flow unit to dry off the surface of the plate and minimize any contamination.

Confirmatory tube tests are grouped together in a test tube rack and agar plates are divided in half with a marking pen. All tubes and agar plates are prenumbered and organized usually in numerical order before any inoculations are begun.

(ii) The transfer of inoculum from a P-A bottle to test tube broths requires a number of steps. If bacterial growth in the P-A bottle has tended to settle to the bottom, slight agitation will redisperse the bacterial growth throughout the growth medium. If the bacterial growth is uniformly distributed throughout the growth medium, agitation will not likely be required. If the inverted tube shows 10% or more gas accumulation, the cap of the bottle should be loosened to relieve any pressure buildup and then retightened before any agitation of the medium takes place.

One or more 3-4 mm inoculating loops should be available to the technician. Just before use, the wire and loop portion of the inoculating loop should be heated in a bunsen burner flame until they are red hot and then allowed to cool. The cap of the P-A bottle should be removed by holding it firmly with the fifth finger of one hand, while the other hand positioned at the bottom of the bottle unscrews the bottle from the cap. The mouth of the P-A bottle is flamed and one of the sterilized inoculating loops is introduced into the mouth of the P-A bottle and lowered until the loop is submerged 10-20 mm below the surface of the medium. The loop is then withdrawn carefully from the P-A bottle (avoid touching mouth or sides of bottle) and the cap is placed back on the mouth of the P-A bottle. While the loop is being held in a horizontal position to avoid losing the inoculum contained in the loop, the test tube being inoculated is removed from the test tube rack and the metal cap is taken off by the fifth finger technique using the same hand that is holding the inoculating loop. The mouth of the test tube is flamed and the inoculating loop is introduced into the mouth of the test tube and lowered until the loop is below the surface of the medium (avoid touching the sides of the test tube). The loop is dipped several times below the surface of the medium before withdrawing the loop from the test tube. The mouth of the test tube is flamed again; the cap is replaced; and the test tube is placed back in its rack again and slanted or turned in a manner to indicate that it has been inoculated. The loop is then flamed until it is red hot being careful to avoid any splattering of inoculum remaining on the loop. This procedure is repeated for all tubes being inoculated or if more than one loopful of inoculum is taken from the same P-A bottle

(iii) The streaking of agar plates to obtain isolated colonies of bacteria requires the following procedure. A smaller inoculating loop (i.e. 2-3 mm) is preferred for streaking agar plates than the one used for inoculating broth tubes. The loop is sterilized in a bunsen burner flame just prior to use. The cap of the P-A bottle is removed aseptically; the inoculating loop is introduced into the P-A bottle; submerged in the broth medium; withdrawn and held in a horizontal position as previously described; and the cap is returned to the P-A bottle.

The bottom portion of the petri dish (containing the agar medium) is picked up with the thumb and fingers of the hand adjacent to the ends of the line drawn to divide the area of the petri dish in half. The petri dish is held at an angle of 45° to the vertical, while the inoculating loop streaks the inoculum on the agar surface. The inoculum is deposited on one half of the petri dish in three equal sections. Using a gentle sweeping motion and non-overlapping streaks, the major portion of the inoculum is deposited in the upper right hand section; the plate is turned 90° and the middle section of the upper half of the plate is streaked; the plate is turned 90° and the upper left hand (third) section of the plate is streaked. The petri dish bottom is returned to the lid and the entire plate is rotated 180° to put it in position to streak the second half of the plate with another culture. The inoculating loop is sterilized in the flame as before. This procedure is repeated for all plates being inoculated from the same P-A bottle

(iv) All tubes and agar plates have observations taken on them within a 24-hour period. In some instances longer incubation periods are required, but observations are taken after each 24 hours of incubation. Only on weekends are results of plate and tube inoculations read after 48 or 72 hours' incubation. A blue- ball point pen is used for marking 24-hour observations; a red pencil for 48-hour observations; a regular lead pencil for 72-hour observations; and a green ball-point pen for 96-hour observations.

c) Types of confirmatory media and their reactions following inoculation for the Coliforms - Aeromonas series of tests.

(i) EC broth - two tubes of this medium are inoculated from a P-A bottle; one tube is incubated at 35°C and another tube is incubated at 44.5°C, both for a 24-48 hour incubation period. A positive result is scored if gas production is evident in the inverted tube after incubation.

If gas is produced in the 44.5°C tube, a MacConkey agar plate is streaked. If gas is produced in the 35°C tube, but no lactose fermenting (pink or red) colonies are found on the MacConkey plate, another MacConkey agar plate is streaked from the 35°C EC tube. If bacterial growth occurs in the EC tubes, but no gas production, the result is indicated by +, if no growth, the result is indicated by -. If no growth occurs in either of the EC tubes, but pink or red colonies occur on the MacConkey agar plate, the inoculation of EC tubes from the P-A bottle should be repeated.

(ii) MacConkey agar plates - a loopful of inoculum from each P-A bottle is streaked on one half of each MacConkey agar plate. The plates are inverted after the streaking procedure and placed in a plastic cakette with a tight-fitting lid. The cakette is then placed in the 35°C incubator and the plates are observed after 24 hours for the presence of bacterial colonies.

The bacterial colonies are viewed with the aid of light from the Daylight Fluorescent Titration Illuminator or other suitable devices. The colonies are described on the bench sheets as to size and colour. The size may range from 0.5 mm to 3-4 mm depending on the growth characteristics of the organism and whether the MacConkey medium has any inhibitory effect on the cells deposited during the streaking procedure. The colonies may appear translucent and the same colour as the medium or they may be opaque with a creamy appearance exhibiting either no colour or various shades of pink through to dark red. They may also be convex or flat with a mucoid or butyrous consistency.

After descriptions of the different types of colonies are noted on the bench sheet, representative colonies are picked off and each is inoculated into an EC broth tube, a Tryptophane tube and onto a Nutrient Gelatin agar plate. Preference is given to pink or red, lactose fermenting colonies before picking non-lactose fermenting colonies. Any non-lactose fermenting colonies less than 0.5 mm in size are generally not picked as they are not considered as potential indicators of sanitary pollution.

MacConkey agar plates incubated for periods longer than 24 hours may produce colonial growth which is unsuitable for designating the type of lactose fermention or for picking colonies to other media. When this occurs, the streaking procedure from the P-A bottle should be repeated.

(iii) EC broth - if the colony selected from the MacConkey agar plate was the result of growth following incubation from a P-A bottle, the EC tube is incubated at 35°C; if the colony selected from the MacConkey agar plate was the result of growth following inoculation from an EC 44.5°C tube, the EC tube is incubated at 44.5°C. A positive result is scored if gas production is evident in the inverted tube after incubation.

(iv) Tryptophane broth - inoculum from the same colony used for the EC tube inoculation is transferred to Tryptophane broth, which is incubated at 35°C for 24-48 hours. After incubation, 0.2 to 0.3 ml of the Kovac's test reagent for indole is added to the tube, which is shaken and observed for a 5-10 minute period for the appearance of a pink to red colour in the reagent layer. This colour reaction constitutes a positive test for indole. A yellow or orange colour may be considered as a negative result.

(v) Nutrient Gelatin agar plate - inoculum from the same colony used for inoculation of the EC and tryptophane broth tubes is used for inoculation of a Nutrient Gelatin agar plate, which is incubated at 35°C for 24-48 hours. After 24 hours, the culture is given an oxidase test by using either the one reagent (a) test system or the two reagent (b) test system.

a) The one reagent test system consists of moistening Whatman #3, 9 cm filter paper with a few drops of the tetramethyl-p-phenylenediamine reagent. Using a flamed, platinum loop, a portion of a bacterial colony is removed from the Nutrient Gelatin agar plate and rubbed onto the moistened portion of the filter paper. Within 10-15 seconds, the bacterial area on the filter paper should turn dark purple or black in colour, if the organisms is oxidase positive. The organism is considered as oxidase negative if, after 60 seconds, no colour change or only a pink or mauve colour has formed

b) The two reagent test system consist of mixing in equal amounts a few drops of reagent A (dimethyl-p-phenylenediamine) with a few drops of reagent B (alpha-naphthol) immediately prior to use. The two reagents are mixed together in a test tube and the mixture is poured onto the Whatman #3, 9 cm filter paper. Using a flamed, platinum loop, a portion of a bacterial colony is removed from the Nutrient Gelatin agar plate and rubbed onto the moistened portion of the filter paper. The formation of a dark purple or black colour within 30-60 seconds in the treated area of the filter paper constitutes a positive oxidase result. If little or no colour change has taken place after five minutes, the organism is considered as oxidase negative

The production of gelatinase will be indicated by a zone of haziness around the bacterial growth in the agar medium within 24-48 hours. A doubtful or negative indication of gelatinase must be checked after 48 hours incubation by flooding the medium surrounding the bacterial growth with a saturated solution of ammonium sulphate $(NH_4)_2SO_4$. This test should be performed only after the oxidase test has been done, as well as the inoculation of an Enterotube, a Phenol Red Dextrose broth, or an Arginine Dyhydrolase tube.

(vi) Enterotubes are inoculated preferably from isolated colonies on the Nutrient Gelatin agar plates. The tubes are incubated at 35°C preferably for 24 and 48 hour periods, but sometimes weekend incubations allow only 48 or 72 hour observations. Results are taken according to the manufacturer's directions.

(vii) Phenol Red Dextrose broth tubes are inoculated at the same time as the Enterotubes using the inoculation wire from the Enterotube. Each tube is incubated at 35°C along with its corresponding Enterotube. Observations are made for acid (yellow colour) production and gas (inverted tube) production.

(viii) Arginine Dihydrolase - this medium is stabbed to the bottom of the tube with inoculum from a colony on the Nutrient Gelatin agar plate using an inoculating needle. Each tube should be overlaid with sterile paraffin oil to a depth of 1 cm. Incubation is at 35°C for 24-48 hours. A marked deepening of the pink colour in the tube indicates a positive test. An uninoculated control tube should be incubated along with the inoculated tube to provide a means of observing a colour change.

d) Types of confirmatory media and their reactions following inoculation for Staphylococcus aureus.

(i) Mannitol Salt agar plate - a loopful of inoculum from each P-A bottle is streaked on one half of a Mannitol Salt agar plate. The plates are inverted after the streaking procedure and placed in a plastic cakette with a tight-fitting lid. The cakette is then placed in the 35°C incubator and examined after 24-48 hours for typical Staphylococcus aureus colonies. These colonies are usually 2-3 mm in size, butyrous and bright yellow in colour. The medium surrounding the colony generally changes from a pink to yellow colour. Cultures other than S. aureus may form white or pink colonies. Occasionally bacillus-shaped organisms will form yellow mucoid colonies on the medium. A Gram stain should be performed on each suspected S. aureus culture and the culture should also be tested for catalase production, coagulase production and fermentation capacity using the OF glucose test.

(ii) Catalase test - this test is done by first placing a glass microscope slide inside a petri dish. A small amount of inoculum from a colony should be placed in the centre of the glass slide. Using a pipetting device, a drop of 3% hydrogen peroxide (H_2O_2) is placed on the inoculum. The pipette is withdrawn immediately and the lid of the petri dish is closed down so that the reaction may be observed through the lid of the petri dish. A positive catalase reaction is indicated by an effervescent or bubbling reaction when the H_2O_2 comes in contact with the inoculum.

(iii) Coagulase test - the culture is inoculated into a tube of Trypticase Soy broth, which is incubated at 35°C for 18-24 hours. About 0.05 ml of the 18-24 hour culture is added to a screw-capped (13 x 100 mm) sterile tube, which has had 0.5 ml of reconstituted rabbit plasma added to it. The tube is gently rotated to ensure a thorough mixing of the contents. The cap is securely tightened on the tube, which is then placed in a 35°C incubator for four hours.

Positive and negative control tubes should be set up at the same time. The tubes will both contain 0.5 ml of rabbit plasma, but one tube will be inoculated with 0.05 ml of a known positive S. aureus culture and the other tube will be inoculated with 0.05 ml of a known negative culture such as E. coli. All tubes are inspected at the end of four hours incubation for clotting of the plasma. If no clotting has occurred, the tubes may be held for another 14 hour period at room temperature. Further incubation may lead to false positive reactions.

Clotting of the known positive S. aureus culture as well as the unknown culture constitutes a positive test for coagulase. Any unused reconstituted plasma may be kept at 4°C for up to 48 hours, if the plasma is kept in a sealed container that prevents evaporation or contamination of the plasma. The known negative culture should preferably be negative for citrate utilization as the plasma may contain citrate.

(iv) OF glucose test - two tubes of this medium should be inoculated by stabbing the tubes almost to the bottom with inoculum from an 18-24 hour Trypticase Soy agar culture One tube is overlaid with 1 cm of sterile paraffin oil. The tubes are incubated at 35°C for 24-48 hours. If the culture is fermentative, both tubes will show a colour change from green to yellow. If the organism is only oxidative, the overlaid tube will show little colour change, whereas the non-overlaid tube will show marked acid production particularly at the surface of the medium. This test would only be required for differentiating Staphylococcus from Micrococcus.

e) Types of confirmatory media and their reactions following inoculation for Pseudomonas aeruginosa.

(i) Drake's broth - following inoculation from the P-A bottle, this medium is incubated at 41.5°C for up to 96 hours. After each 24 hour period, the Drake's broth is checked for evidence of growth and fluorescence using a long wave (366 nm) ultraviolet light. If no fluorescence occurs in the first 24 hours, the tube is reincubated and checked for fluorescence in the subsequent 24 hour periods. When fluorescence occurs, inoculum is streaked from the Drake's broth onto Skim Milk agar and then onto a Nutrient Gelatin agar plate and an Acetamide agar slant from growth on the Skim Milk agar plate.

(ii) Skim Milk agar - on this medium, P. aeruginosa grows well forming light green colonies, which fluoresce under the ultraviolet light, and have a definite clearing zone (1-3 mm) in the medium around the colonial growth area.

(iii) Nutrient Gelatin agar plate - on this medium, P.aeruginosa will be usually positive for gelatinase and oxidase reactions (see c (v))

(iv) Acetamide agar slants - inoculum streaked on the surface of the slant will change the colour of the slant from a pale, straw colour to a deep pink or magenta, if P. aeruginosa is present

(v) In some instances, P. aeruginosa may be detected directly on MacConkey agar plates, because colonies have a grape-like odour, a blue-green colour, and have a flat, irregular appearance. Inoculum from these types of colonies may be streaked directly on Skim Milk agar plates

(vi) Oxy/ferm tubes - colonies which give a positive oxidase test and are positive or negative for gelatinase on Nutrient Gelatin plates should periodically be "identified" by inoculating into Oxy/ferm tubes. These tubes are inoculated in a similar manner as Enterotubes and are incubated at 35°C for 24-48 hours, except over a weekend when only 48 or 72 hour observations may be made. Results of positive and negative tests are scored according to the manufacturer's directions.

f) Types of confirmatory media and their reactions following inoculation for fecal streptococci.

(i) Ethyl violet Azide (EVA) broth - this medium is inoculated from the P-A bottle with 2-3 loopfuls of inoculum. The medium is incubated at 35°C for 24-48 hours. Each day the medium is checked for growth. A positive result is constituted by a dense bacterial suspension. With some cultures, sedimentation of the bacterial suspension may take place giving a blue to purple button-like formation when the tube is viewed from below

(ii) Enterococcus agar plates - a loopful of inoculum from each PA bottle is streaked on one half or one quarter of an Enterococcus agar plate. The plates are inverted after the streaking procedure and placed in a plastic cakette with a tight-fitting lid. The cakette is then placed in the 35°C incubator and examined after 24-48 hours for the presence of typical fecal streptococci colonies. These colonies are small, dark red or maroon in colour, usually about 0.5 to 1 mm diameter. A catalase test should be performed on these colonies to ensure the colonies are catalase negative.

A positive result for fecal streptococci is indicated when typical colonies grow on the enterococcus medium and good growth occurs in the EVA broth. If one or the other of the results is negative, but not both, inoculum should be transferred from the medium which gave a positive result to the medium which gave a negative result. If, after the appropriate incubation period, the result is still negative, an overall negative result is given for the presence of fecal streptococci. If the catalase test is positive, the result for fecal streptococci is marked as negative

g) Types of confirmatory media and their reactions following inoculation for Clostridium perfringens.

(i) Skim Milk tube - with this test, anaerobic conditions must be established in the tube prior to inoculation. Each Skim Milk tube is placed in a beaker of water filled so that the water level in the beaker is the same height as the medium level in the Skim Milk tube. The beaker of water is brought to a slow boil and kept there for five minutes. The tubes are removed from the boiling water and allowed to cool in a test tube rack until they can be held comfortably by hand (usually about 45°C).

Inoculation of the medium consists of taking a sterile one ml pipette; removing the cap from the P-A bottle; placing the pipette almost to the bottom of the P-A bottle; carefully draw up one ml of inoculum; withdraw the pipette keeping the forefinger securely over the top of the pipette to prevent the inoculum from escaping; replace the cap on the P-A bottle; remove the cap from the Skim Milk tube; place the pipette down to the bottom of the tube; by manipulating the pipette in a rolling fashion with the fingers allow 0.1 ml of inoculum to escape from the pipette; remove the pipette from the Skim Milk tube; replace the cap on the tube; place the pipette and any remaining inoculum into a plastic waste disposal jar containing a dettol or Wescodyne solution

The Skim Milk tube is then placed in a test tube rack and incubated at 35°C for 24-48 hours. Each day the tube is examined for clotting of the skim milk and the formation of copious amounts of gas which tear holes in the clotted milk giving a reaction known as a "stormy fermentation". An inoculating loop is introduced into the tube and a loopful of inoculum is transferred to a clean glass slide; allowed to dry; and the slide is given a Gram stain. If examination of the slide shows the presence of short, plump, rectangular-shaped Gram positive rods with square ends and occurring as single cells or in short chains, these cells are typical for Clostridium perfringens.

The absence of a "stormy fermentation" in the Skim Milk tube and/or the absence of typical C. perfringens cells seen during the microscopic examination is considered as a negative test

5.) C. Gram Stain Procedure

a) The preparation of Gram stained slides is required for

(i) all presumptive positive P-A bottles

(ii) Skim Milk tubes having a "stormy fermentation"

(iii) suspected Staphylococcus aureus cultures from Mannitol Salt agar plates

b) Slides prepared for examination of inoculum from bottles should first be marked with an identification number on the frosted glass portion of the slide using a lead pencil. Sometimes, four to six cultures may be put on one slide by using a red, wax, china-marking pencil to section the slide off into compartments. Two loopfuls of broth inoculum from each P-A bottle are placed in each compartment. The second loopful of inoculum is placed on top of the first loopful and no attempt is made to spread out the inoculum. The inoculum in each compartment is allowed to evaporate to dryness and only then is the slide heat-fixed by passing the inoculated areas on the slide through a bunsen burner flame several times until the glass slide is quite warm, but not too hot to be placed on the back of one's hand

c) Slides prepared from "stormy fermentation" Skim Milk tubes are done in a similar manner to 5 (b) above, however, the inoculum should be taken from the "whey" portion of the Skim Milk rather than the coagulated portion. Otherwise, the coagulated milk will contribute to difficulties in preparation of properly stained bacterial cells

d) Slides prepared from suspected S. aureus colonies on Mannitol Salt agar plates should first have one or two drops of water placed on the microscope slide. Using a flamed inoculating needle, a small portion of bacterial growth is removed from the same colony as that receiving the catalase test. The inoculating needle is tapped gently into the drop of water on the slide to allow a suspension of bacteria to form in the water. The inoculating needle is flamed and allowed to cool before using it to mix and spread the bacterial suspension into a thin film on the microscope slide. Precautions should be taken to avoid creating too dense a bacterial suspension, which could produce a slide which is difficult to read and interpret. The inoculating needle is flamed again

e) All slide preparations are air-dried and heat-fixed before beginning the staining procedure. The slides are then taken over to a sink area and placed on a staining rack

f) Crystal violet stain is applied to each slide and allowed to cover the area(s) on the slide which contain the bacterial growth. The crystal violet is left in contact with the bacterial cells for one minute. During the above staining period, the cold water tap is turned on to provide the minimum flow of water from the tap that can be obtained without changing to a series of drops. After one minute, each slide is placed in the stream of water about two inches from the orfice of the tap and the crystal violet is quickly washed from the slide

g) The slide is placed back on the staining rack and the iodine solution is immediately added to the slide to cover the stained area(s) on the slide. The iodine solution is left in contact with the slide for a minimum period of one minute. If more than one slide is stained at the same time, the iodine solution may be left on the slide indefinitely until each other slide is decolourized and counterstained without any adverse effects.

After one minute, the iodine solution is quickly, but gently washed from the slide and excess water is blotted off the slide with a paper towel. The slide is now ready for the decolourizing step

h) After the excess water is blotted from the slide and before the slide has become dry, the 95% alcohol decolourizer is applied to the stained area(s) and the slide is tilted slightly back and forth to allow the alcohol to wash the stained area(s) of the slide. After 30 seconds, the alcohol is rinsed from the slide under the tap water in about 4 to 5 seconds

i) The safranin counter stain is quickly added to the slide for a period of about one minute and then it is also quickly washed from the slide. Excess water is blotted from the slide with a paper towel and the slide is allowed to air-dry before making the microscopic examination

j) The microscopic examination is done by placing the slide on the microscope stage and positioning the slide so that the light is passing through one of the stained areas on the slide. With one of the low power objectives in place, the microscope is focused until the stained area on the slide is sharply in focus. The objective lens is moved out of position and a drop of immersion oil is placed in the centre of the stained area. The immersion oil lens is moved into position and using the fine adjustment knob, the bacterial cells are carefully brought into focus.

If trouble is experienced in focusing the bacterial cells, the slide should be moved until one of the red, wax markings on the slide is brought into view. The red wax marking on the surface of the slide is brought into sharp focus and the slide is carefully moved back towards the stained area of the slide while the viewer attempts to keep the surface of the slide in focus. Care must be taken that sufficient oil is present on the slide to allow this manipulation to take place.

k) For viewing and interpreting the Gram stain reaction of bacterial cells, the technician should endeavour to locate an area on the slide in which the bacterial cells are not clumped together but evenly dispersed. Bacterial cells which are deep purple or violet should be considered as Gram positive. Bacterial cells which are red or pink will be Gram negative. Difficulties in determining the Gram reaction of bacterial cells may be resolved somewhat if the light intensity is kept down to a minimum. If this does not work, a repeat Gram stain should be made on a 24-hour plate or broth culture.

5.) D. Procedure - Isolation and Confirmation of Sheen-Colonies from Membrane Filters on m-Endo Agar LES

a) All drinking water samples, which have had a MF analysis as well as P-A analysis and which have produced sheen colonies on the membrane filter plate, will have one or more of the sheen colonies checked for coliform confirmation.

b) Following enumeration of the sheen and background colonies, the membrane filter plate will be examined for the different sheen colony types, which will be described on the tally sheet as to size and sheen intensity. Colonies selected for isolation should be well isolated from other surrounding colonies.

c) Using a recently flamed, but cooled inoculating needle, the tip of the inoculating needle is gently touched to the top of the sheen colony to allow adherence of some of the colony on the tip of the needle. (The technician should avoid sticking the needle down through the colony until it touches the membrane filter or attempting to obtain the whole colony on the end of the inoculating needle as other viable but non-multiplying bacteria may be picked up and transferred to the new growth medium.)

d) The inoculating needle containing the inoculum is held in one hand, while the other hand retrieves a previously marked EC tube from the test tube rack. The hand holding the inoculating needle between the thumb and forefinger is used to remove the cap from the EC tube using the fifth finger to hold the cap firmly during the inoculation process. The top of the EC tube is flamed and the inoculating needle is introduced into the test tube. The tip of the inoculating needle containing the inoculum is immersed below the surface of the EC broth 2-3 times and the needle is withdrawn from the test tube. (Avoid touching the sides of the test tube above the medium during the inoculation procedure.) The cap is replaced on the EC tube, which is then placed back in the test tube rack. A second EC tube is withdrawn from the test tube rack and the inoculation procedure is repeated. The inoculating needle is then carefully flamed to avoid spattering of the inoculum. One of the EC tubes is incubated at 35°C and the other is incubated at 44.5°C for 24-48 hours.

e) After 24 hours incubation, EC tubes showing growth and gas production are streaked on MacConkey agar plates. This includes tubes incubated at both 35 and 44.5°C. If gas production is not evident, the tubes are incubated an additional 24 hours. If after the additional 24 hours growth but no gas production is observed in the tubes, only the 35°C tube is streaked on a MacConkey agar plate. MacConkey agar plates are incubated for 24 hours at 35°C.

f) Following incubation, colonies growing on MacConkey agar are described on the tally sheet as to size and colour. Representative colonies are picked off and each is inoculated into an EC broth tube, a Tryptophane broth tube and a Nutrient Gelatin agar plate. Preference is given to pink or red, lactose

fermenting colonies before picking non-lactose fermenting colonies. Any non-lactose fermenting colonies less than 0.5 mm in size are generally not picked as they are not considered as potential indicators of sanitary pollution

g) The procedures involved in incubating and taking observations on the inoculations into EC broth, Tryptophane broth and Nutrient Gelatin agar will be the same as that described in Section 5B -c (iii - v). Additional tests done on cultures will follow the methodology described in 5B -c (vi - viii).

h) The interpretation of the results of confirmatory tests on sheen colony isolates will be similar to that for organisms isolated from P-A bottles and will be found in Section 6.

6.) Interpretation of presumptive, confirmatory and the additional test analysis results

a) Differentiation and identification of pollution indicator bacteria isolated from P-A bottles depends on evaluation of the results of the confirmatory and additional test analyses. With most of the bacterial groups, one or more analyses must give a positive result before the particular bacterial group is indicated as being present in the original water sample. What constitutes a positive result for each of the respective tests has been described in Section 5B - c, d, e, f and g.

b) The total coliform group is marked as present, if the EC 35°C broth tubes show growth and gas production; lactose fermenting colonies are present on the Mac Conkey plate; and the colony growth on Nutrient Gelatin agar is oxidase negative.

c) The fecal coliform group is marked as present, if the EC 44.5°C broth tubes show growth and gas production; lactose fermenting colonies are present on the MacConkey agar plate; and the colony growth on Nutrient Gelatin agar is oxidase negative.

d) The anaerogenic coliform group is marked as present, if the EC 35°C broth tubes show growth but no gas production; lactose fermenting colonies are present on the MacConkey agar plate; and the colony growth on Nutrient Gelatin agar is oxidase negative.

e) The Aeromonas group is marked as present, if colony growth on the Nutrient Gelatin agar is gelatinase positive and oxidase positive. The EC 35°C broth tubes will usually show growth and no gas production. Colonies on MacConkey agar plates may or may not show lactose fermentation. The tests for indole production and arginine dihydrolase should be positive.

f) The fecal streptococcus or enterococcus group is marked as present, if the EVA broth tube is positive for growth and typical dark red colonies which appear on the Enterococcus agar plate are catalase negative

g) Pseudomonas aeruginosa is marked as present, if the Drake's medium exhibits fluorescence; typical blue-green colonies with a grape-like odour are formed on the MacConkey agar plate; the Skim Milk agar has colonies with a green pigment and with hydrolysis of the casein evident around the colonies; the colonies are oxidase positive; the acetamide slant shows growth and the presence of acetamide deaminase by a magenta or purple colouring of the slant; and gelatinase is produced.

h) Clostridium perfringens is marked as present, if the Skim Milk tube shows a "stormy fermentation" and typical C. perfringens cells are observed in a Gram stain.

i) Staphylococcus aureus is marked as present, if butyrous bright yellow colonies form on Mannitol Salt agar plates; a Gram stain shows typical Gram positive cocci; a catalase test is positive and a coagulase tube is positive.

j) If one or more of the positive characteristics for any pollution indicator group cannot be demonstrated, that group should be considered absent from the sample. P-A bottles, which have doubtful, conflicting or negative results for any of these groups, will have the tally sheet marked with a "presumptive only" result, which is marked by "P".

k) Any cultures, which have been inoculated into either Enterotubes or Oxy/ferm tubes from which a generic identification has been made, will have the identification name specified on the tally sheet. In some instances, results from these tubes may permit amending any results which have previously been designated as "P" on the tally sheet.

PRESENCE-ABSENCE (P-A) TEST SCHEME